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TITLE: Does the Androgen Receptor (AR)-Regulated Map Kinase Phosphatase 1 (MKP-1) Enhance Castration-Resistant Prostate Cancer Survival under Therapeutic Stress?

PRINCIPAL INVESTIGATOR: Russell Szmulewitz

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Russell Szmulewitz

E-Mail: rszmulew@uchicago.edu

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University of Chicago
5841 S. Maryland Avenue,
MC 2115
Chicago, IL 60637

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14. ABSTRACT

Metastatic castration resistant prostate cancer (mCRPC) is clinically treated with both taxane chemotherapy and androgen pathway modulators. Identification of a mediator of resistance across therapy classes is a critically unmet need and would be a significant innovation in the field. Map Kinase Phophatase 1 (MKP-1, DUSP1) is a known regulator of the stress activated protein kinase cascade that can inhibit the activity of pro-apoptotic Map Kinases JNK and p38. It has an established anti-apoptotic, pro survival role, and has been implicated in chemotherapy resistance in breast cancer models, and is inversely associated with apoptosis in preclinical prostate cancer models. Androgen and glucocorticoid signaling can induce MKP-1 expression; as mCRPC remains driven by androgen receptor signaling, and as mCRPC is often treated adjunctively with corticosteroids, MKP-1 may be a down stream effector of prostate cancer cell survival that facilitates therapy resistance. The work proposed sought to test the hypothesis that MKP-1 plays a role in the development of therapy resistance, independent of therapeutic class, and thus, if inhibited, would potentiate the effects of both hormonal and chemotherapies.

To date, significant progress has been made including optimization of MKP-1 protein detection methodologies and the generation of highly bone metastatic CRPC cell lines that can be traced over time with bioluminescence imaging. Ongoing experiments are being undertaken with MKP-1 over expression and knock-down within these cell lines to test for *in vivo* therapy resistance.

15. SUBJECT TERMS
Metastatic castration resistant prostate cancer (mCRPC), Prostate Cancer (PC), Enzalutamide (Enza), Map Kinase Phophatase 1 (MKP-1)

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Table of Contents

1 INTRODUCTION:

Metastatic castration resistant prostate cancer (mCRPC) is clinically treated with both taxane chemotherapy and androgen pathway modulators. Identification of a mediator of resistance across therapy classes is a critically unmet need and would be a significant innovation in the field. Map Kinase Phophatase 1 (MKP-1, DUSP1) is a known regulator of the stress activated protein kinase cascade that can inhibit the activity of pro-apoptotic Map Kinases JNK and p38. It has an established anti-apoptotic, pro survival role, and has been implicated in chemotherapy resistance in breast cancer models, and is inversely associated with apoptosis in preclinical prostate cancer models. Androgen and glucocorticoid signaling can induce MKP-1 expression; as mCRPC remains driven by androgen receptor signaling, and as mCRPC is often treated adjunctively with corticosteroids, MKP-1 may be a down stream effector of prostate cancer cell survival that facilitates therapy resistance. The work proposed sought to test the hypothesis that MKP-1 plays a role in the development of therapy resistance, independent of therapeutic class, and thus, if inhibited, would potentiate the effects of both hormonal and chemotherapies.

2 KEYWORDS

The following are key words that will be used in this report:
Metastatic castration resistant prostate cancer (mCRPC)
Prostate Cancer (PC)
Enzalutamide (Enza)
Map Kinase Phophatase 1 (MKP-1)

3 ACCOMPLISHMENTS:

A. What were the major goals of the project?

Objectives: To demonstrate that decreased MKP-1 expression enhances response to androgen targeted therapy (Aim #1) and docetaxel chemotherapy (Aim #2) and delays therapy resistant progression in a preclinical model of metastatic CRPC.

As described in the statement of work, these were the outlined goals of the project

Task 1. **Generation and optimization of cell lines**

a. Develop prostate cancer cell lines lines stably expressing luciferase construct for use in bioluminescence
b. Develop cell lines that express doxycycline inducible MKP-1 knockdown
c. Confirm that cell lines, when exposed to doxycycline *in vitro* display knockdown of MKP-1

Task 2: Establish progressive mCRPC *in Vivo*

a. Perform intracardiac injections to disseminate prostate cancer cells systemically
b. Follow animals for development of metastatic disease using live animal bioluminescence imaging
c. Castrate animals upon development of metastatic disease
d. Follow for the development of castration resistance by bioluminescence imaging

Task 3: Treatment with MDV3100, docetaxel chemotherapy with/without MKP-1 knockdown

Upon development of castration resistance, separate mice into two cohorts-one for MDV3100 hormonal treatment and one for docetaxel treatment

a. Treat animals with docetaxel or MDV3100 for 4 weeks. Half the mice will also receive diet containing doxycycline to induce MKP1 knockdown
b. Monitor metastatic disease progression with bioluminescence while on treatment until disease endpoint

Task 4: Pathologic evaluation of metastatic tissues:

a. Harvest tissue from euthanized mice
b. Send to tissue resource center for fixation/processing/staining
c. Evaluate the tumor tissue for histologic characterization, staining for proliferation and apoptosis.

B. What was accomplished under these goals?

Although not all of the tasks were completed (see Section 5), significant progress was made on many of the tasks.

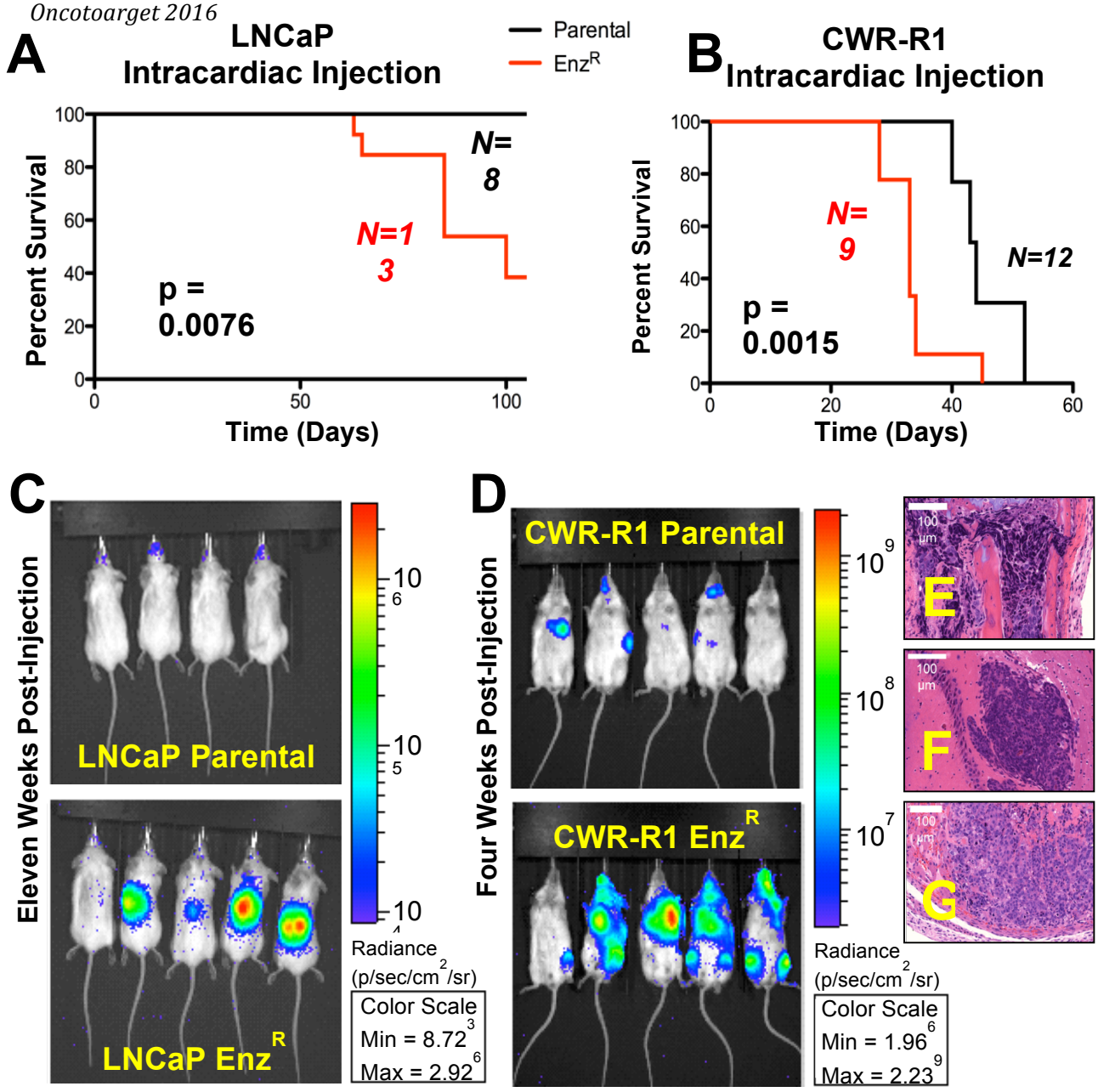
Task 1/Task 2 focused on the development of luciferase-expressing cell lines for use in the experiments. They are both complete.

The cell lines initially proposed (C4-2B and CWR 22Rv11) were utilized for our metastatic model initially. However, the C4-2B cell line generated no distant metastases subsequent to intracardiac cell inoculation. The 22Rv1 cell line did establish metastases, however the metastatic yield was low with this line. We therefore optimized new cell lines for our experimental metastasis assay. Two cell lines were generated to be resistant to the androgen receptor antagonist enzalutamide (Enza-R). Enza-R LNCaP cells and CWR-R1 cell lines demonstrate significant castration resistant metastatic progression (Figure 2). Of note, the C4-2 line is a derivative of the LNCaP line, and the CWR-22Rv1 and CWR-R1 lines are similarly genetically related. Thus although the cell lines we are utilizing are different from those proposed, they are genetically and phenotypically very similar. Task 2 has therefore been completed.

The baseline MKP-1 RNA/protein expression for our cell lines of interest has been assessed (Figure 2). Notably, the endogenous protein expression of MKP-1 protein is very low in these cell lines, even under hormonal stimulation with both Dexamethasone (GR agonist) and R1881 (AR agonist).

As the endogenous protein expression is low in these cell lines, knocking down MKP-1 expression was not pursued. As an alternative, MKP1 overexpression was undertaken. The initial MKP-1 over-expression construct that we used (see report from 2015) failed to express wild-type MKP-1 protein and in fact had a mutation within the open reading frame of unknown phenotypic consequence. We therefore obtained the wild-type open reading frame and cloned it into the LV105 expression vector. Overexpression was confirmed.

Figure 1. Metastatic model optimization. Enzalutamide-resistant (Enza-R) cell lines were derived through prolonged culture in enzalutamide-containing media. 5 x 10^5 (LNCaP, A) or 2.5 x 10^5 (CWR-R1, B) parental or Enza-R luciferase expressing cells were inoculated via intracardiac injection. Animal survival was significantly shorter with Enza-R compared to parental cells for both lines. Metastatic disease was more frequent and with higher volume as denoted by bioluminescence (C, D). Representative histology of bone (E), brain (F) and adrenal gland (G) CWR-R1 Enza-R metastases. *Modified from Kregel et al, Oncotoarget 2016*



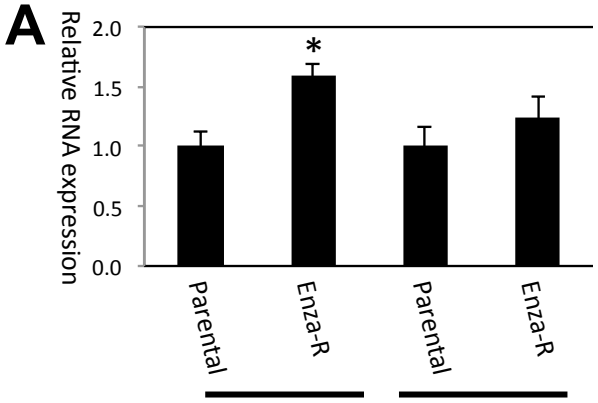
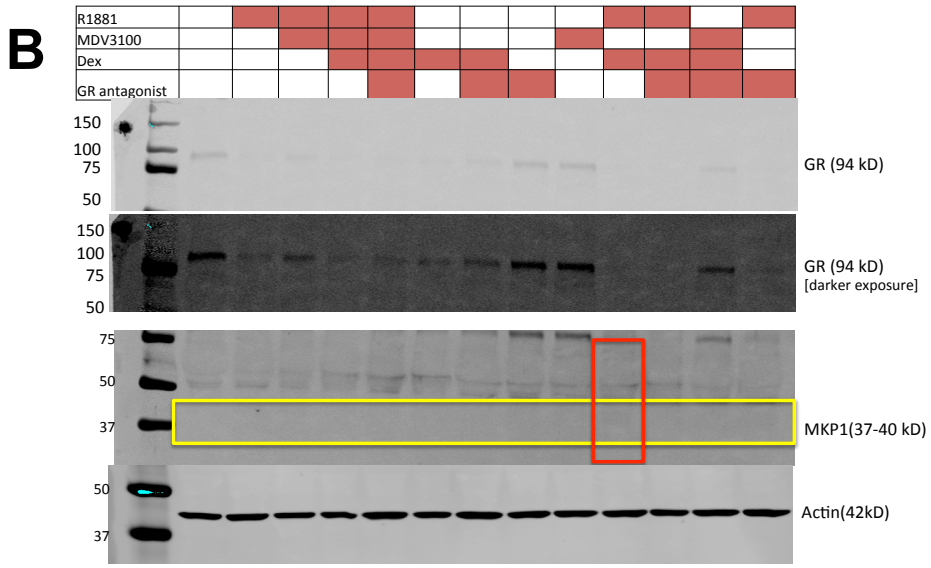
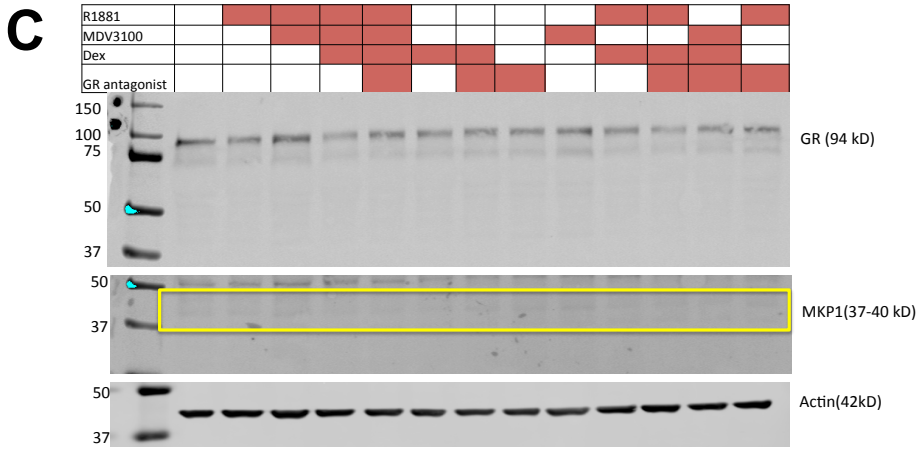


Figure 2. Endogenous MKP-1 expression in PC lines. A. Endogenous *MKP-1* RNA expression by qRT-PCR showing increased expression in Enza-R CWR-R1's (* P<0.05) and stable levels in LNCaP despite enzalutamide treatment. B. MKP-1 protein in LNCaP-Enza-R induced with dexamethasone (Dex) and androgen (R1881) showed slight increase (red box); however MKP-1 expression by protein is very low in these cells (yellow box). C. MKP-1 protein in LNCaP-Enza-R MKP-1 expression by protein is very across conditions (yellow box).





Task 3 involved interrogation of the cell lines for sensitivity to both hormonal (enzalutamide-MDV3100) and chemotherapy (docetaxel) as the hypothesis was that increased MKP-1 expression would impart chemotherapy and hormonal therapy resistance. We interrogated the MKP-1 overexpressing, luciferace expressing enzalutamide-resistant cell lines from Task 1 and 2. Contrary to the stated hypothesis, we found no enhanced therapy resistance with MKP-1 over-expression (Figure 3). As there was no phenotype *in vitro* imparted with MKP-1 overexpression, further *in vivo* studies with these cell lines were not pursued as was deemed unethical use of animal resources.

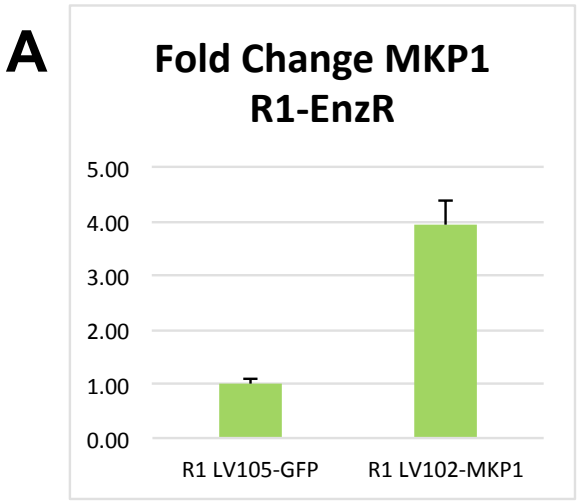
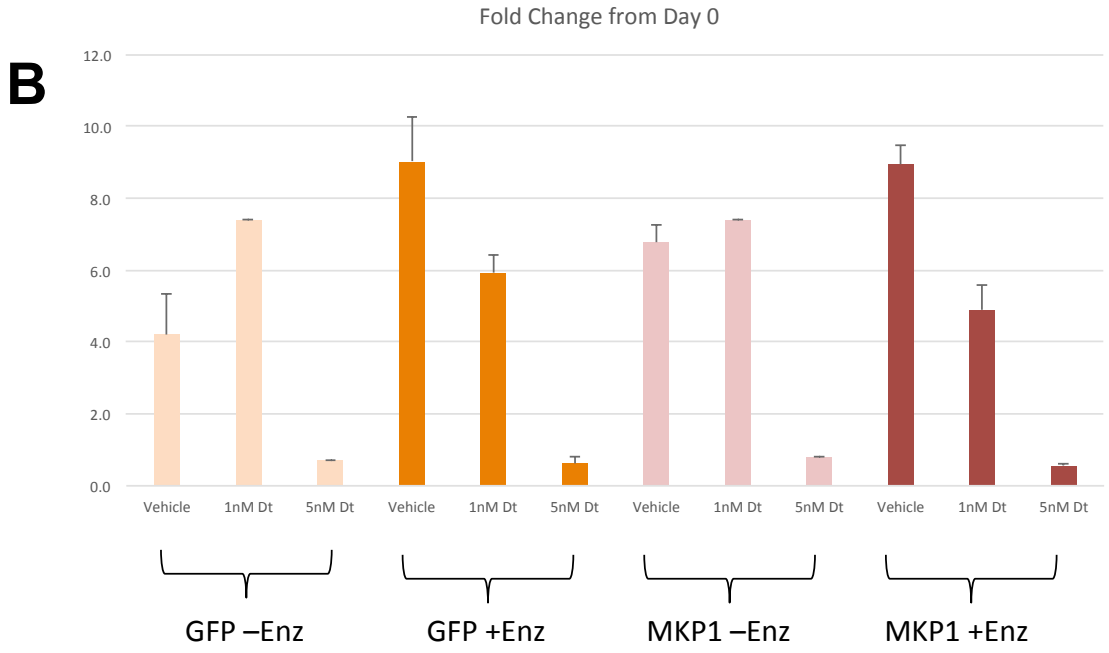


Figure 3. MKP-1 over-expression does not impart therapy resistance. A. *MKP-1* RNA expression is increased by qRT-PCR within the MKP-1 over-expressing Enza-R CWR-R1's (* P<0.05). B. Cell viability of MKP-1 over-expressing Enza-R CWR-R1's (Red shaded bars) was compared to GFP-expressing controls (orange bars). Both lines grew in enzalutamide, with no increase with MKP-1 (dark orange "vehicle" compared to maroon "vehicle"). MKP-1 also failed to impart resistance to Docetaxel (Dt) at 1 or 5nM as single agent (-Enz) or along with enzalutamide.



For Task 4, as we felt *in vivo* exploration was unwarranted given *in vitro* findings, the bulk of the work outlined in Task 4 will not be pursued However, as prostate cancer is often bone metastatic, we have optimized a metastatic model that spread within the bones (Figure 1). As a necessary byproduct of this model, we have needed to optimize models for identifying, obtaining and decalcifying bone metastases for histopathologic examination within this task. This has been accomplished (Figure 1 for example) and has been reported (Kregel et al, *Oncotarget*, 2016, *in press*).

C. What opportunities for training and professional development has the project provided?

The work within this award allowed the PI to develop a set of new, clinically relevant prostate cancer cell lines that metastasize to the bone. In doing so, the PI was able to refine skills necessary to both engineer the cell lines and also perform robust metastasis assays. More importantly, the PI had the opportunity to manage both post doctoral

fellows and students during the course of this work. This experience improved the PI's leadership skills.

D. How were the results disseminated to communities of interest?
The Enza-R cell line data has now been published in *Oncotarget* (Kregal et al, 2016, *in press*) with the PI as co-final author.

E. What do you plan to do during the next reporting period to accomplish the goals?
The funding period has been completed (one year award with no-cost extension). No further work on this project will be undertaken; however a rotating summer student will compile the MKP-1 data for manuscript submission.

4. IMPACT:

A. What was the impact on the development of the principal discipline(s) of the project?
The generation of Enza-R cell lines that are highly metastatic, especially to the bone, is a novel and highly impactful contribution to the prostate cancer field. We have already been asked by other investigators for these lines and are sharing them with the community. We also published our bone histology techniques, which will have an impact on the prostate cancer field. The MKP-1 related findings were largely negative (contrary to the stated hypothesis) and thus are unlikely to have a major impact on the field.

B. What was the impact on other disciplines?
Surprisingly, endogenous MKP-1 protein analysis by Western blot has not been routinely reported in the literature, likely due to difficulties with commercially available antibodies. We have optimized techniques using one of these antibodies, which increases specificity and sensitivity of endogenous MKP-1 protein detection. This will have an impact beyond the prostate cancer field for others studying MKP-1 once reported. In addition, we have engineered new lentiviral MKP-1 over-expression constructs that may be used in other research disciplines.

C. What was the impact on technology transfer?
None

D. What was the impact on society beyond science and technology?
None

5. CHANGES/PROBLEMS:

A. Changes in approach and reasons for change
As noted above, we changed the prostate cancer cell lines have used in this work. The reasons for this change are noted above, but restated, the initially selected cell lines did not reproducibly produce a metastatic phenotype, despite their report in the literature. The cell lines we are using now reproducibly metastasize. Another change involves the use of MKP-1 over-expressing cell lines. We initially proposed conditional MKP-1 knockdown, however, as endogenous MKP-1 protein expression is very low, we engineered cell lines to over-express flag-MKP-1.

B. Actual or anticipated problems or delays and actions or plans to resolve them Delays/problems were reported within the previous report. In addition, the initial MKP1 over-expression construct we received from a colleague and initially used during this reporting period had a mutation in the open reading frame. This necessitated the generation of a new lentiviral over-expression construct.

C. Changes that had a significant impact on expenditures

Although we did not pursue *in vivo* experiments with the MKP-1 over expressing cells, the generation of novel cell lines that metastasize to the bone *in vivo* resulted in neural expenditures.

D. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents: None
E. Significant changes in use or care of human subjects: None
F. Significant changes in use or care of vertebrate animals: None
G. **Significant changes in use of biohazards and/or select agents:** None

6. PRODUCTS:

A. Publications, conference papers, and presentations:
The following manuscript is in press and contains data generated from this award:

Steven Kregel, James L. Chen, Westin Tom, Venkatesh Krishnan, Jacob Kach, Hannah Brechka, Tim B Fessenden, Masis Isikbay, Gladell P. Paner, **Russell Z. Szmulewitz**[*], and Donald J. Vander Griend[*] (* Authors contributed equally). Acquired Resistance to the Second-Generation Androgen Receptor Antagonist Enzalutamide in Castration-Resistant Prostate Cancer. *Oncotarget*. 2016. *In Press.*

B. Website(s) or other Internet site(s): NA
C. Technologies or techniques: Technique for optimization of MKP-1 protein detection was developed and will be reported in next manuscript. A new metastatic model was developed (see above). A new MKP-1 over-expressing lentiviral construct has been engineered.
D. Inventions, patent applications, and/or licenses: NA
E. Other Products: NA

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

A. What individuals have worked on the project?
Other then the principal investigator, the postdoctoral fellow (acob Kach) have worked on the project. In addition, a graduate student, Steve Kregel, has been peripherally involved in the project, assisting with live animal imaging and in vivo studies. The animal experiments were performed in collaboration with Dr. Donald Vander Griend.

B. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? The PI is the PI for major project within a newly funded SPORE in prostate cancer that provides new support for the investigator.
C. What other organizations were involved as partners? None

8. SPECIAL REPORTING REQUIREMENTS

None

9. APPENDICES

None